POSTURAL ORTHOSTATIC TACHYCARDIA SYNDROME AND GASTROINTESTINAL SYMPTOMS: Contribution of Gastrointestinal Peptides

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1. Background

Postural Tachycardia Syndrome (POTS) is a disabling condition that mostly affects young women in their reproductive age. It is characterized by chronic (>6 months) orthostatic intolerance symptoms (palpitation, lightheadedness, blurred vision and mental clouding) triggered by assuming an upright posture and that improved upon recumbency. These symptoms are associated with a rapid increase in heart rate (≥30 bpm) that occur within 10 minutes upon standing (1-2). POTS is estimated to affect up to 3 million persons in the United States (3) and is considered a syndrome rather than a single disease.

The pathophysiology of POTS is complex, and are related to abnormal cardiovascular autonomic adaptation to postural changes. Under normal conditions, the assumption of upright posture does not result in major changes in blood pressure due to the integration of complex autonomic, circulatory and neurohumoral responses. Upright posture-induced a fluid shift of approximately 700 mL of blood from the upper thorax to the splanchnic circulation and lower extremities, which result in decrease in venous return to the heart, ventricular filling, and stroke volume. These changes cause unloading of the arterial baroreceptors and increase in sympathetic activity, vasoconstriction and restoration of stroke volume and cardiac output.

In POTS patients, multiple mechanisms have been proposed to explain the exaggerated increase in heart rate. The orthostatic tachycardia could be a compensatory phenomenon to hypovolemia, impaired sympathetic-mediated vasoconstriction or increased vascular compliance. The later could induce an exaggerated fluid shift upon standing from thorax to lower body. Depending on the mechanism involved different POTS phenotype has been described: (i) hypovolemic POTS; (ii) neuropathic POTS; and (iii) POTS associated with Ehlers-Danlos and joint hypermobility syndrome (EDS/JHS) (4). Of note, there is overlapping in the pathophysiology of POTS with patients having more than one etiology, **Fig.1**.

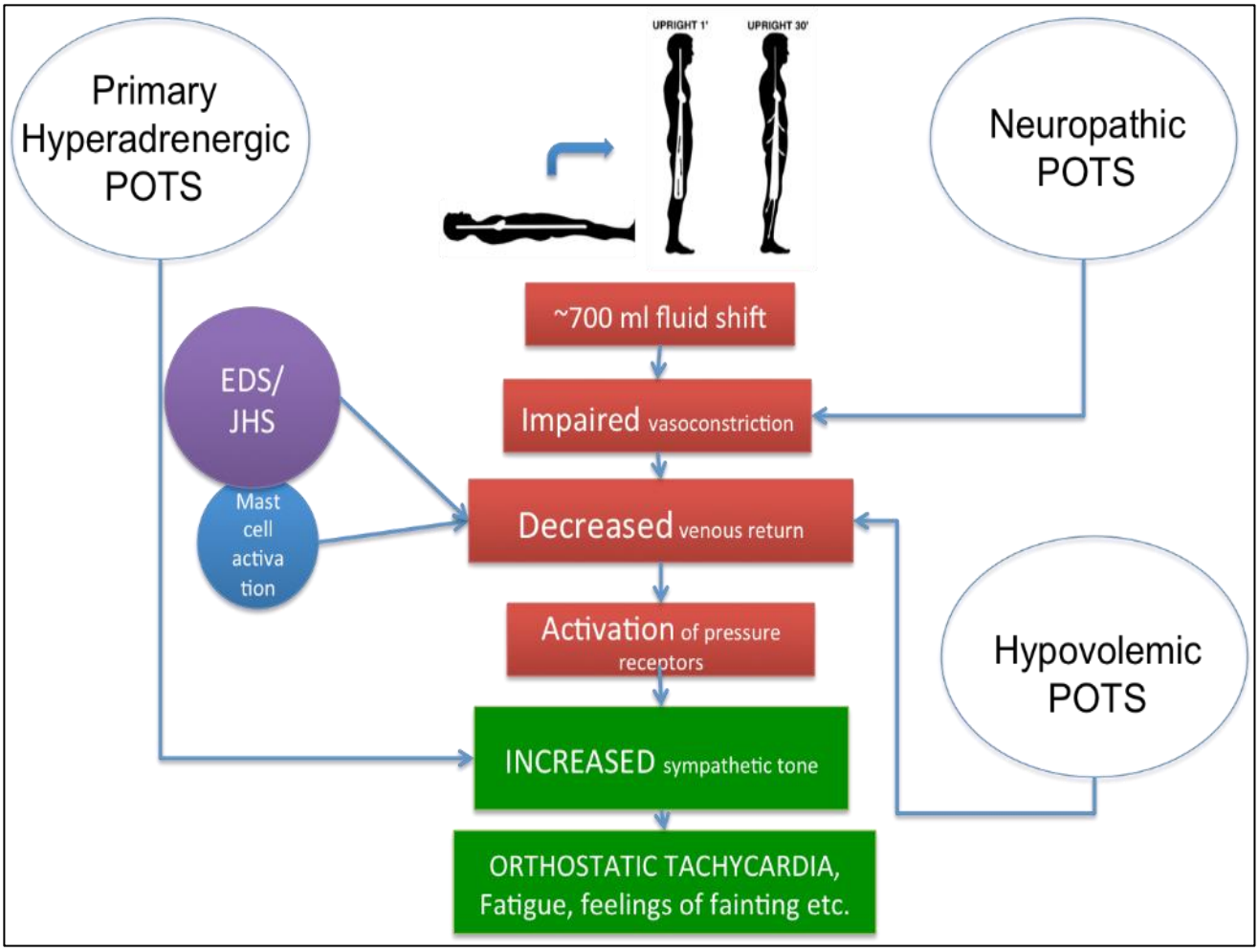

Fig.1. Pathophysiology of POTS and its different phenotypes. Arrows showed specific areas affected by different POTS phenotypes. EDS/JHS, Ehlers Danlos Syndrome/Joint Hypermobility Syndrome.

POTS and gastrointestinal symptoms

In addition to the cardiovascular symptoms, patients with POTS experience significant gastrointestinal symptoms namely nausea, bloating, diarrhea or even severe constipation. Furthermore, large meals or high carbohydrate meals exacerbates the feelings of palpitations, weakness, and fatigue in these patients (5).

Multiple studies have reported the presence of alterations in the gastrointestinal motility. Table 1 summarized all the published report, pooled data from 352 patients recruited from 6 different studies, showed 21-80% prevalence of nausea, vomiting, and abdominal pain. In four of these studies that measured gastric motility, they found that 43% prevalence of rapid gastric emptying and 20% prevalence of delayed gastric emptying. Furthermore, Al-Shekhlee et al. reported a high prevalence of impaired sudomotor function in the POTS patients who reported GI symptoms suggesting that abnormal post-ganglionic sympathetic

function could play a role in the pathophysiology of these GI abnormalities. Interestingly, although gastrointestinal complications have already reported in all types of EDS (6), recent study which made more attention on functional non-life-threatening has shown GI symptoms in hypermobility EDS are more common compare to other types of disease (7).

Table 1: Prevalence of gastrointestinal symptoms in POTS

Author(s)	Population (N)	Gastrointestinal Symptoms	GI motility studies
Lawal et al. (6)	61 POTS (all with mild to moderate abnormal CASS*)	49 (80%) Nausea 28 (46%) Vomiting 54 (67%) Abdominal pain	27 (44.2%) Rapid 17 (27.9) Delayed 17 (27.9) Normal
Antiel et al. (7)	21 POTS (24% with abnormal sudomotor function)	15 (71%) Nausea 10 (47%) Vomiting 14 (67%) Abdominal pain	5 (24%) Rapid 4 (19%) Delayed 12 (57%) Normal
Wang et al. (8)	28 POTS	24 (86%) Nausea 11 (40%) Vomiting 20 (70%) Abdominal pain	NA
Loavenbruck (9)	163 POTS (36% with abnormal sudomotor function)	34 (21%) Nausea 16 (10%) Vomiting	78 (48%) Rapid 30 (18%) Delayed 5 (34%) Normal
Park et al. (10)	22 POTS (32 % with abnormal sudomotor function)	18 (82%) Nausea or vomiting 8 (59%) Abdominal pain	6 (27%) Rapid 2 (9%) Delayed 14 (63%) Normal
Al-Shekhlee A et al. (11)	19 neuropathic-POTS /38 non-neuropathic POTS	13 (68%)/10 (26%) combine symptoms#	NA

* CASS: Composite autonomic scoring system
#Abdominal pain, bloating, nausea, and constipation

2. Evidence of autonomic neuropathy in POTS patients

We previously defined a subgroup of POTS patients in whom we detected a partial peripheral autonomic neuropathy primarily affecting lower extremities (a.k.a. neuropathic POTS). These subjects had decreased norepinephrine spillover in response to sympathetic activation (8) and abnormal sweat volumes and prolonged latency detected by quantitative sudomotor axon reflex (QSART)(9). Recently, Gibbons and Freeman (2013) strengthen the definition by providing histological evidence of neuronal damage with the inclusion of skin biopsies with specific staining for autonomic dense fiber and sensitivity assessment (10).

In Neuropathic POTS there is evidence of impaired vasomotor tone in different specific vascular bed, particularly the splanchnic circulation (11, 12). Tani *et al.* reported reduced

splanchnic vascular resistance and increase in resting mesenteric blood flow providing evidence of splanchnic denervation (13).

In summary, there is evidence of post-ganglionic sympathetic denervation is a subset of patients with POTS. The most current definition are based on the presence of abnormal sudomotor and sensitivity assessment.

3. Gastrointestinal Peptides and Autonomic Regulation

The sympathetic nervous system (SNS) provide innervation to the enteric ganglia, the circular muscles of sphincters, and the mucosa of the stomach and intestines (14). The SNS also negatively regulate the motor and secretory functions of the gastrointestinal (GI) tract. Browning and Travagli (2014) reported that the absence of sympathetic inhibitory innervation causes excessive and uncoordinated activity in the GI tract (15). Indicating that a preserved ANS regulation of the GI tract is crucial for the maintenance of normal GI motility.

In addition to regulating the motor function, the SNS and parasympathetic nervous system (PNS) regulate the postprandial GI peptides secretion by enteroendocrine cells (EEC) (16). EECs are the first line components of the Brain-Gut axis (17). Multiple peptides, such as incretins (GLP-1, GLP-2, GIP), and PYY are important for the maintenance of glucose homeostasis. They are secreted by a different type of EEC (17) in the GI tract. Prior to their absorption, nutrients in the GI lumen are important stimuli for peptide secretion in the ileum in rats (18), pigs (19), and humans (20). These peptides are secreted before the bulk of ingested meal reaches to the ileum, suggesting the presence of a neuronal/endocrine pathway in GI tract (21, 22).

In summary, the SNS through innervation the gut smooth muscle; ENS and EECs negatively regulate the GI motor function and incretins secretion which impact glucose homeostasis.

4. Effect of autonomic denervation in the GI tract on Gut Peptide Secretion

Evidence from animal models showed that when rats underwent removal of the superior autonomic mesenteric ganglia that contains mostly SNS neurons and were challenged with an oral glucose gavage; plasma insulin and C-peptide secretion were increased compared with controls (non-gangliectomised rats). Furthermore, glucose levels were much lower in the gangliectomised rats suggesting that the SNS splanchnic innervation plays a critical role in the maintenance of glucose homeostasis (23). The increased secretion of insulin and C-peptide levels in this model could be explained by an increase in incretin hormonal release.

Additional studies using isolated guineas pig ileum (*in vitro* model) showed that GLP-1 secretion is inhibited by SNS nerve stimulation which is mediated by α-adrenergic receptors (24).

In summary, in the absence of sympathetic tone on ENS and EECs the incretins secretion increases which may cause low levels of plasma glucose.

5. Hypothesis

The focus of the present proposal is to determine glucose homeostasis, GI motility, and their association with GI and cardiovascular symptoms in POTS patients versus healthy controls. Furthermore, we will determine differences in these outcomes in POTS patients with and without evidence of postganglionic sympathetic fiber neuropathy.

6. Inclusion/Exclusion Criteria

Inclusion Criteria

- 18-60 years old
- Postural Tachycardia Syndrome: Heart rate increase >30 bpm from supine within 10 min of standing, in the absence of orthostatic hypotension (>20/10 mmHg fall in blood pressure), with chronic symptoms (> 6 months), and in the absence of other acute cause of orthostatic tachycardia.
- Able and willing to provide informed consent
- Female premenopausal subjects must utilize adequate birth control and willingness to undergo serum beta-hCG testing

Exclusion Criteria

- Use of acetaminophen or acetaminophen-related drugs (over-the-counter) in the 24 hours prior to the study.
- Hypertension (>150 mmHg systolic and >100 mmHg diastolic) based on history or findings on screening.
- Orthostatic hypotension (consistent decrease in BP >20/10 mmHg with 10 min stand)
- Pregnancy
- History of type 1 or type 2 diabetes mellitus

- Cardiovascular disease, such as myocardial infarction within 6 months, angina pectoris, significant arrhythmia (sinus tachycardia is not excluded), deep vein thrombosis, pulmonary embolism
- History of serious neurologic disease
- Impaired hepatic function (aspartate amino transaminase and/or alanine amino transaminase >1.5 x upper limit of normal range)
- Impaired renal function (serum creatinine >1.5 mg/dL)
- Hematocrit <28%
- Any underlying or acute disease requiring regular medication that could possibly pose a threat to the subject or make implementation of the protocol or interpretation of the study results difficult
- Inability to comply with the protocol

Healthy control subjects

Defined as subjects without any significant past medical history, non-smokers, and on no chronic medications at the time of the study. Healthy control subjects will be age- and BMI-matched to the POTS patients.

Positive control

Patients with complete autonomic neuropathy (pure autonomic failure) will be enrolled as positive control. This condition is defined as complete autonomic failure based on AFT (autonomic function test) and norepinephrine plasma levels less than 100 pg/ml.

7. Enrollment/Randomization

The participants with POTS and "complete autonomic failure" will be recruited from patients referred to the Vanderbilt University Autonomic Dysfunction Center. Additional patients will be recruited from the POTS registry in ResearchMatch, and information about the study will be posted on websites associated with POTS support groups. Healthy volunteers will be recruited from a population of previous participants in autonomic studies, through the ResearchMatch.org database, Subject Locator, and through advertising and emails around the Vanderbilt community.

When contacting appropriate study candidates, the investigator will describe the complete protocol. The participants will then be given a written informed consent form that has been approved by the Vanderbilt Institutional Review Board. The subject will be given adequate

time to read the consent form, ask questions, and if satisfied by the responses, sign the form. Consent or refusal to participate in this study will not affect medical care. No modifications or waiver of the elements of consent will be necessary for the execution of this study. Consent procedures will take place in the Autonomic Dysfunction Center at Vanderbilt University Medical Center. We will use e-consenting. We will review medical record of patients.

8. Study Procedures

Screening day: Subjects will undergo history and physical, screening laboratory tests (CBC, C-peptide, insulin, BMP), posture study, supine and standing plasma norepinephrine with blood specimen collection, and autonomic reflex testing. If the patients have had routine laboratory measurements within the past 3 months, we will not repeat them. If the patient has had autonomic reflex testing, supine and standing plasma norepinephrine performed in our center, we will not repeat them, and we will use the existing data.

Patients will be examined for hypermobility Ehler-Danlos syndrom (type III EDS or h-EDS). The diagnosis is made clinically. According to 2017 diagnosis criteria, all three of the following should be present:

1. Generalized joint hypermobility

2. Two or more of following features:

   A. Systemic manifestations of a more generalized connective tissue disorders
   B. Positive family history
   C. Musculoskeletal complications

3. All of following prerequisites required:
   A. Absence of unusual skin fragility
   B. Exclusion of other heritable and acquired connective tissue disorders
   C. In patients with an acquired/autoimmune connective tissue disorder additional diagnosis of h-EDS requires meeting both features A and B of criterion 2. Feature C of criterion 2 (chronic pain and/or instability) cannot be counted towards a diagnosis of h-EDS
   D. Exclusion of alternative diagnoses that may also include joint hypermobility by means of hypotonia and/or connective tissue laxity.

The posture study involves patients lying down for at least 15 min (to allow for a supine steady state), heart rate (HR), SBP and DBP (Dinamap, Critikon Corp) will be measured. The patient then stands for 10 minutes (as tolerated) with HR, SBP, and DBP measured at 1, 3, 5, and 10 min standing. Orthostatic changes will be calculated as the difference between supine parameters and measurements obtained at the end of standing. Patients unable to stand for 10 minutes will be allowed to sit for the 10 min before blood is sampled.

In the afternoon, we will do **autonomic function tests** to determine how well the autonomic nervous system is working in regulating blood pressure and heart rate. For these tests, we will measure heart rate using a continuous electrocardiographic trace, blood pressure using a cuff around one arm and/or finger. The autonomic function tests include asking the subject to breathe deeply for two minutes and breathing rapidly for 30 seconds, maintaining a handgrip for 3 minutes, blowing against pressure for 15 seconds, and placing one hand in ice water for 1 minute. All these tests are meant to stimulate the autonomic nervous system to produce changes in blood pressure and heart rate of short duration that reflect how well the involuntary nervous system is working.

Total blood drawn for the screening day will be 45 ml.

Study day: The study will be conducted as case-control design. Each group will undergo a modified oral glucose tolerance test (OGTT) as described in section 9. In addition, we will assess GI symptoms and hemodynamics before and after oral glucose (at minute 0, 30, 60, 90, and 120). Blood sampling (20 ml) will be done at baseline and after 5, 10, 15, 30, 60 ,90, and 120 minutes for measuring of plasma levels of GI peptides (GLP-1, GLP-2, PPY, glucagon, C-peptide, insulin). Gastric emptying will be evaluated by acetaminophen absorption test (AAT). An additional sample (30 ml) will be collected at baseline for serum to be used for LPS, LBP, sCD14, and I-FABP (as GUT cells damage markers), total bile acids, and short-chain fatty acids (SCFA). Total blood drawn for the study day will be 190 ml.

Fecal samples will be collected at home by participants in 5-ml conical tubes containing media to stabilize RNA for microbiome study and bile acid and SCFA assay.

Figure 2. Experimental Protocol - Study Day

		1.4 grams acetaminophen															END
		75 grams glucose oral															
Time, min	**BL (-10)**	**5**	**10**	**15**	**20**	**25**	**30**			**60**			**90**			**120**	**125**
Evaluation																	
Blood samples	X	X	X	X			X			X			X			X	
GI symptom survey	X						X			X			X			X	

Orthostatic vital signs	X							X			X			X			X
POTS-related symptoms	X							X			X			X			X

Sub-study 1
We will collect fecal microbiome and dietary data from an additional 60 subjects (30 positive controls and 30 health controls). These procedures will be the same as for the main study, however these subjects will not participate in other study procedures.

Sub-study 2
We will invite all POTS patients who completed the primary study to self-administer a questionnaire to assess for Chronic Fatigue Syndrome. The questionnaire will include the Checklist Individual Strength (CIS), the Chalder Fatigue Scale, and a Vanderbilt University Medical Center Autonomic Dysfunction Center Chronic Fatigue Screen.

Sub-study 3
In this sub-study, we hypothesize that 75g oral glucose will increase upright splanchnic venous capacitance in POTS patients, but not in controls. We will invite all patients who completed the primary study to come to the Autonomic Dysfunction Center for a 2-day study.

On Day 1, we will ask the patient to rest supine on a tilt table for splanchnic venous capacitance measurements. The subjects will then be asked to drink a 75gr glucose solution and will undergo 75-degree head-up tilt at 30, 60, 90, and 120 minutes. At each timepoint, we will repeat the measurement of splanchnic venous capacitance.

On Day 2, we will place an abdominal binder on the subjects and inflate it to 40mm Hg to counteract the effect of oral glucose on the splanchnic venous capacitance. We will then repeat the Day 2 procedures.

9. Specific procedures

Oral glucose tolerance test (OGTT): In the case of OGTT, subjects will be given a ready-to-use test solution containing 75 g glucose dissolved in 300 mL water, immediately after fasting blood sampling. They will be instructed to drink the test solution within 5 mins. Blood samples will be drawn at 5, 10, 15, 30, 60, 90, and 120 minutes after drinking the ready-to-use test solution, Fig.1. Gastric emptying will be measured by acetaminophen absorption test.

Acetaminophen absorption test (AAT): Acetaminophen (20 mg/kg) will be given to patients. Serum acetaminophen will be determined by fluorescence polarization

immunoassay. This assay uses a six-point calibration curve, and the detection limit is 4 μmol/L. The coefficient of variation is less than 5%. Estimation of the rate of gastric emptying was based on serum concentrations of acetaminophen in the blood samples collected. An algorithm that transforms serum concentrations of paracetamol into estimates of gastric emptying was applied. This algorithm adjusts for first-pass metabolism, unequal distribution and individual rate of elimination, and provides estimates for the percentage of meal emptied from the stomach as a function of time.

Gastrointestinal symptoms scoring: The 2-page questionnaire is based on elements from a questionnaire that have been validated with some modifications (25). The questionnaire contains 17 questions on the frequency of GI symptoms that have been troublesome in the preceding 6 months. The frequency of each symptom is rated on seven-point Likert scale from no discomfort to very severe discomfort.

Hemodynamic symptoms scoring: Hemodynamic symptoms will be measured by using the Vanderbilt POTS Symptom Score. The patients will be asked to rate the severity of 9 symptoms on a 0–10 scale (with 0 reflecting an absence of symptoms). The sum of the scores at each time point will be used as a measure of symptom burden. The 9 symptoms are: mental clouding, blurred vision, shortness of breath, rapid heartbeat, tremulousness, chest discomfort, headache, lightheadedness, and nausea. This symptom score has been previously used by our center, and the symptoms were chosen as they reflect common complaints of patients with POTS.

Glucose and insulin levels: Glucose levels will be measured with a glucose analyzer (YSI Life Sciences, Yellow Springs, OH).

GI peptides measurements: The plasma designated for GLP-1 measurement will be supplemented with aprotinin (1,000 KIU/ml) and dipeptidyl peptidase-4 inhibitor (20 μl/ml plasma; Millipore, St. Charles, MO). Plasma insulin, c-peptide, glucagon, GIP, active GLP-1 (7-37 and 7-36 amide), peptide YY, pancreatic polypeptide, and leptin were measured by multiplex immunoassays (Luminex xMAP, Millipore).

Total bile acid assay: Total bile acid measurement in plasma and feces samples will be measured by LC/MS in the Vanderbilt core facility.

Short-chain fatty acids assay: Short chain fatty acids will be measured by GC/MS method in the Vanderbilt core facility.

Fecal microbiome assay: Fecal microbiome will be established by shotgun 16s metagenomic sequencing. At least 0.5g of fecal sample will be collected by participants at home (OMNIgene Gut sample collection kit). Samples will be stored in media designed to stabilize RNA and refrigerated until sent to VANTAGE.

Dietary data collection: Data on food intake of participants will be collected two weeks prior to the fecal sample collection by using Health watch 360 Research Portal (gbhealthwatch.com). This portal is designed for academic research for collecting diet data using mobile app technology. Participants will be invited by email and enrolled one by one or batch upload.

Splanchnic venous capacitance measurements: While segmental bioimpedance is monitored, continuous positive airway pressure (CPAP) will be applied sequentially at 0, 4, 8, 12 and 16 cm H2O for about 30 seconds each; this positive airway pressure will increase the intrathoracic pressure, which is transmitted to the venous circulation. Pressure (CPAP pressure, x-axis) - volume (splanchnic vascular volume measured by segmental impedance and expressed as percent change from baseline, y-axis) relationships are then constructed to assess for splanchnic venous capacitance.

Statistical Considerations:

The primary endpoints are GLP-1, GIP, and PYY. Secondary endpoints include GI symptom scores, orthostatic symptoms scores, diversity of gut microbiome, and plasma levels of gut cell damage markers.

Sample size calculation:

This is a pilot study; our primary endpoint will be the AUC in GLP 10-120 minutes after a 75-gram oral glucose challenge. There are no available data on the response in patients with POTS to an oral glucose tolerance test. In a previous study using a 14-gram glucose challenge, the AUC in GLP-1 was 50.4±29.8 pg/ml/min. Based on studies in animal models, we expect a 24% (24) difference between POTS and controls, the estimated sample size would be 100 subjects per group with probability (power) 0.8 and alpha=0.05.
We will enroll 35% (26) of this estimated samples size for this pilot study - 35 patients with POTS, 35 healthy controls, and 35 positive controls (patients with complete autonomic failure).

Statistical Analysis Plan:

This is a pilot study; we will enroll a total of 105 subjects. Data obtained from this pilot study will allow us to measure the variability in our measurements and to calculate a sample size for future studies investigating the glucose hemostasis and GI peptide secretion in POTS patients.

We will use standard graphing and screening techniques to detect outliers and to ensure data accuracy. We will assess continuous outcomes for normality. If normality is violated, we will apply data transformation or consider non-parametric analysis methods. We will provide summary statistics for both numerical and categorical variables by study groups. We will estimate between-group difference in means with its 95% confidence interval (CI) for the primary endpoints. These differences will be tested using either two-sample t-test or Wilcoxon Rank Sum test. Important variables will be assessed for their association with the study endpoints and their between-group difference. This will help us to identify confounders. We will conduct additional regression analyses using general linear models (GLM) to evaluate group differences (patients with POTS, patients with neuropathic POTS, healthy controls, and positive controls) while controlling for confounders and adjusting for important covariates, such as gastric emptying While the small sample size of this pilot study may limit our ability to conduct these regression analyses to avoid over-fitting the model, we will conduct these analyses in the full study. Analyses of secondary endpoints and other exploratory analyses will be conducted similarly. We will test all hypothesis at the level of α=0.05. We will use SPSS for Windows (Version 24.0, SPSS, Chicago) and the open source statistical package R (version 3.1.0, R Core Team, 2014) for analyses (30).

10. Risks

Blood Draws: Subjects may experience discomfort, bruising, and/or bleeding, or infection at the needle insertion site after a blood draw. Rarely, some people faint.

12-hour fast: Participants may experience hunger during a 12-hr fast, however this is a standard requirement by clinicians for accurate blood testing.

QSART: Subjects may experience a mild sensation of low electrical current at the site during iontophoresis.

OGTT: The glucose solution may not taste good.

Genetic Testing:
There is a risk to the participant's privacy if someone gains access to the genetic data we have collected in the event of a security breach. If the samples or results are linked to the

participant's name, it could cause problems with insurance or getting a job. There also may be other privacy risks that we have not foreseen.

For individuals who provide authorization for their blood samples to be shared with other investigators for future research use: Although their name and other information that is traditionally used to directly identify them (such as name or address) will never be stored with their sample for this project, people may develop ways in the future that would allow someone to link their genetic information back to them (or a blood relative) in another database if they have participated in other genetic testing studies. The risk of this happening, however, is very rare.

It is possible that important medical information may be uncovered as part of the routine laboratory tests and physical exam (such as elevated blood pressure, diabetes, or elevated liver enzymes). If important information is uncovered, it will be discussed with the subject. The subject may be encouraged to discuss these findings with their primary care physician. Should the need for immediate medical attention arise, resources at Vanderbilt University Medical Center will be utilized.

Breach of Confidentiality

In the event of a security breach, confidential information may be stolen. We have strong security procedures in place to minimize the possibility of a breach. Although we cannot provide a 100% guarantee that the participant's data will be safe, our procedures minimize the chance that a breach will take place.

11. Reporting of Adverse Events or Unanticipated Problems involving Risk to Participants or Others

Adverse event grading and attribution scale:

A serious adverse event (AE) is defined as either fatal or life-threatening, requires inpatient hospitalization or prolongation of an existing hospitalization, results in persistent or significant disability/incapacity, is medically significant, or requires intervention to prevent one or more of the outcomes listed above.

All adverse events will be graded according to the following scale:

0 = No adverse events or within normal limits
1 = Mild; did not require treatment

2 = Moderate; resolved without treatment
3 = Severe; required professional medical attention
4 = Life-threatening or disabling
5 = Death

Attribution scale for AE reporting:

- ***Probable:*** the Adverse event is related to the procedures (blood draws), including pain, bleeding, perforation of contiguous organs, infection, and death, if death resulted from one of the aforementioned complications.
- ***Possible:*** Adverse event follows the procedures within a reasonable period (within 7 days), but may have been produced by the subject's clinical state or other factors.
- ***Remote:*** Adverse event does not follow the procedures within a reasonable period (more than 7 days) and could readily have been produced by the subject's clinical state or other factors.
- ***Unrelated:*** the adverse event is judged to be clearly due to extraneous causes and does not meet the above criteria.

AE reporting:

During each study, the subject will be monitored continually and closely. If any adverse events occur, they will be evaluated as follows:

- The clinical research coordinator is responsible for collecting and recording all clinical data. As results are collected, all adverse events will be identified and reported to the PI or co-investigator within 24 hours. The PI is responsible for evaluating each adverse event.
- All unanticipated, serious adverse events related to the experimental procedures that are Grade 3 or above will be reported to the IRB within 7 days after the PI is made aware of the event. The PI will review all Grade 0-2 adverse events.
- All protocol deviations will be reported to the IRB on an annual basis.
- The annual summary of all adverse events and any audit reports will be sent to the IRB.

12. Study Withdrawal/Discontinuation

At any time, subjects may withdraw from the study at their own request. A subject's participation in the study may be discontinued at any time at the discretion of the Investigator and in accordance with her clinical judgment. No disadvantage will arise for

any subject who withdraws consent for participation at any time or who is withdrawn from the study by the Investigator.

Reasons for discontinuation of study treatment may include the following:

- Subject's request for withdrawal
- Investigator's decision that discontinuation is in the best interest of the subject
- Non-compliance with the regimen and timing that might result in dropping out from the study
- Development of an intolerable adverse event due to study participation as determined by the investigator, subject, or both
- Development of an intercurrent illness, condition, or procedural complication, which would interfere with the subject's continued participation

13. Privacy/Confidentiality Issues

Consent will be obtained by a member of the research team in a private location. The confidentiality of participant data will be assured by the use of a study ID number (a combination of the patient's initials and a number). Only members of the research team will have access to research information, PHI, or the key for ID numbers. All electronic data will be maintained in HIPAA-compliant, password-protected databases. Paper documents will be stored in the locked office of the study coordinator and research nurse. All plasma and serum samples will be de-identified before being sent for analysis.

Members of the research team will have access to the patient's medical record during the screening visit and throughout the study until the patient completes participation in the study or meets any of the criteria for study withdrawal/discontinuation. Research records will be maintained for at least three (3) years from the date the study is closed. All PHI documentation will be maintained for at least six (6) years from the date of the last use. After this period, records will be maintained indefinitely by the principal investigator.

It is possible that important medical information may be uncovered as part of the routine laboratory tests and physical exam (such as elevated blood pressure, diabetes, or elevated liver enzymes). If important information is uncovered, it will be discussed with the subject. The subject may be encouraged to discuss these findings with their primary care physician. We will not have a certificate of confidentiality.

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